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**PROVISIONAL APPLICATION FOR  
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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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For: IDENTIFICATION OF COMPOUNDS FOR OVERCOMING  
MUTATION-MEDIATED DRUG RESISTANCE

1. 29 sheets of specification.
2. 8 sheets of drawings
3. Please charge the required application filing fee of \$160.00 (large entity), and any other fees that may be required, to the deposit account of **Kenyon & Kenyon**, deposit account number **11-0600**. A duplicate of this sheet is enclosed.
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4. This invention was not made by an agency of the United States Government or under a contract with an agency of the United States Government.

Dated: May 23, 2004

Respectfully submitted,

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## IDENTIFICATION OF COMPOUNDS FOR OVERCOMING MUTATION-MEDIATED DRUG RESISTANCE

### BACKGROUND OF THE INVENTION

[0001] The progressive development of drug resistance in a patient is the hallmark of chronic treatment with many classes of drugs, especially in the therapeutic areas of cancer and infectious diseases. Molecular mechanisms have been identified which mediate certain types of drug resistance phenomena, whereas in other cases the mechanisms of acquired or *de novo* resistance remain unknown today.

[0002] One mechanism of induced (acquired) drug resistance originally thought to be relevant in the area of cancer therapy involves increased expression of protein known as P-glycoprotein (P-gp). P-gp is located in the cell membrane and functions as a drug efflux pump. The protein is capable of pumping toxic chemical agents, including many classical anti-cancer drugs, out of the cell. Consequently, upregulation of P-glycoprotein usually results in resistance to multiple drugs. Upregulation of P-glycoprotein in tumor cells may represent a defense mechanism which has evolved in mammalian cells to prevent damage from toxic chemical agents. Other related drug resistance proteins have now been identified with similar functions to P-gp, including multidrug-resistance-associated protein family members such as MRP1 and ABCG2. In any event, with the advent of the development of compounds that are specific for a given target protein, and less toxic, the importance of P-glycoprotein and related ATP-binding cassette (ABC) transporter proteins in clinically significant drug resistance has lessened.

[0003] Another possible molecular mechanism of acquired drug resistance is that alternative signal pathways are responsible for continued survival and metabolism of cells, even though the original drug is still effective against its target. Furthermore, alterations in intracellular metabolism of the drug can lead to loss of therapeutic efficacy as well. In addition, changes in gene expression as well as gene amplification events can occur, resulting in increased or decreased expression of a given target protein, and frequently requiring increasing dosages of the drug to maintain the same effects. (Adcock and Lane, 2003)

[0004] Mutation induced drug resistance is a frequently occurring event in the infectious disease area. For example, several drugs have been developed that inhibit either the viral reverse transcriptase or the viral protease encoded in the human immunodeficiency (HIV) viral genome. It is well established in the literature that repeated treatment of HIV-

infected AIDS patients using, for example, a reverse transcriptase inhibitor eventually gives rise to mutant forms of the virus that have reduced sensitivity to the drug which resulted from mutation is that have occurred in the gene encoding reverse transcriptase that render the mutant form of the enzyme less affected by the drug.

[0005] The appearance of drug resistance during the course of HIV treatment is not surprising considering the rate at which errors are introduced into the HIV genome. The HIV reverse transcriptase enzyme is known to be particularly error prone, with a forward mutation rate of about  $3.4 \times 10^{-5}$  mutations per base pair per replication cycle (Mansky et al., *J. Virol.* 69:5087-94 (1995)). However, analogous mutation rates for endogenous genes encoded in mammalian cells are more than an order of magnitude lower.

[0006] New evidence shows that drug resistance can also arise from a mutational event involving the gene encoding the drug target (Gorre et al. *Science*, 2001; PCT/US/02/18729). In this case, exposure of the patient to a specific therapeutic substance such as a given cancer drug that targets a specific *protein-of-interest* (POI, or "target" protein) may be followed by the outgrowth of a group of cells harboring a mutation occurring in the gene encoding the protein that is the target of the therapeutic substance. Whether the outgrowth of this population of cells results from a small percentage of pre-existing cells in the patient which already harbor a drug-resistant mutation in the POI, or whether such mutations arise *de novo* during or following exposure of the animal or human being to a therapeutic agent capable of activating or inhibiting said POI, is presently unknown. In either case, such mutation events may result in a mutated protein (hereinafter defined as a *theramutein*) which is less affected, or perhaps completely unaffected, by said therapeutic substance.

[0007] Chronic myelogenous leukemia (CML) is characterized by excess proliferation of myeloid progenitors that retain the capacity for differentiation during the stable or chronic phase of the disease. Multiple lines of evidence have established deregulation of the Bcr-Abl tyrosine kinase as the causative oncogene in certain forms of CML. The deregulation is commonly associated with a chromosomal translocation known as the Philadelphia chromosome, which results in expression of a fusion protein, p210<sup>Bcr-Abl</sup>, which has tyrosine kinase activity. Transformation appears to result from activation of multiple signal pathways including those involving RAS, MYC, and JUN. Imatinib mesylate (also termed STI-571, "Gleevec") is a 2-phenylamino pyrimidine that targets the ATP binding

site of the kinase domain of Abl (Drucker et al, NEJM 2001, p. 1038). Subsequently it has also been found by other methods to be an inhibitor of platelet-derived growth factor  $\beta$  receptor, and the Kit tyrosine kinases, which are involved in the development of gastrointestinal stromal tumors (see below).

[0008] Until recently, it had not been observed that mutation of an *endogenous* gene during the course of treatment with a specific inhibitor of the corresponding cellular protein would lead to expression of protein variants whose cellular functioning was resistant to the inhibitor. Work by Charles Sawyers and colleagues (Gorre et al., Science 293:876-80 (2001); see also PCT/US/02/18729) demonstrated for the first time that treatment of a patient with a drug capable of inhibiting the p210<sup>Bcr-Abl</sup> tyrosine kinase, i.e. STI-571 (Gleevec) could give rise to mutations in the gene encoding the p210<sup>Bcr-Abl</sup> cancer causing target protein which contains the Abelson tyrosine kinase domain. Such mutations gave rise to mutant forms of p210<sup>Bcr-Abl</sup> which were less responsive to Gleevec treatment than was the original cancer causing version. Notably, the mutations that arose conferred upon the mutant protein a relative resistance to the effects of the protein kinase inhibitor drug, while maintaining a certain degree of the original substrate specificity of the mutant protein kinase. Prior to Gorre et al.'s work, it was believed by those skilled in the art that the types of resistance that would be observed in patients exposed to a compound which inhibited the Abelson protein kinase, such as STI-571 (imatinib mesylate; Gleevec), would have resulted from one of the other mechanisms of drug resistance listed above.

[0009] Accordingly, the ability to treat clinically relevant resistant mutant forms of proteins that are otherwise the targets of an existing therapy, is would be very useful. Such mutated proteins (theramuteins) are beginning to be recognized and understood to be important targets in recurring cancers, will become important in other diseases as well. However, there exists a need for the identification of therapeutic agents that are active against such variant forms of cellular proteins that arise during or following drug therapies, as well as a substantial need for the development of technologies and methods capable of identifying and characterizing such therapeutic agents. Therefore, a key purpose of this invention is to provide methods which may be utilized to identify and pharmacologically characterize inhibitors or activators of any theramutein. An additional purpose of this invention is to provide compounds that may serve as potential therapeutic agents useful in overcoming mutation-induced drug resistance in endogenously occurring proteins.

**BRIEF SUMMARY OF THE INVENTION**

[0010] This invention relates to agents that are inhibitors or activators of variant forms of endogenous proteins, and to novel methods of identifying such agents. Of particular interest are inhibitors and activators of endogenous protein variants, encoded by genes which have mutated, usually following exposure to a chemical agent which was an inhibitor or activator of the corresponding unmutated endogenous protein. Such mutant proteins, herein termed "theramuteins," occur either spontaneously in an organism or arise as a result of selective pressure which results when the organism is treated with a given chemical agent capable of inhibiting the non-mutated form of said theramutein, herein termed a "prototheramutein."

[0011] The invention also provides a general method that can be used to identify substances that will activate or inhibit a theramutein, to the same extent, and preferably to an even greater extent than a known drug substance is capable of inhibiting the corresponding "wild type" form of that protein. (The skilled artisan is well aware, however, that said "wild type" forms of such proteins may have already mutated in the course of giving rise to the corresponding disease in which said protein participates.)

[0012] The invention provides for a fundamentally new way of treating cancer and other diseases where treatment with an existing drug compound, by whatever mechanism, is followed by identifiable (clinically significant) theramutein-mediated drug resistance, by providing alternative drugs that can be administered as theramuteins arise (Wakai et al.; 2004), or preemptively before the outgrowth of clinically significant populations of theramutein expressing cells. Further, where a drug treatment for a particular disease is less effective in a subset of individuals that express a certain theramutein of a protein that the drug targets, the invention enables the tailoring of treatments provided to those subjects by providing alternative drug substances.

[0013] The invention provides a method of determining whether a chemical agent is at least as effective a modulator of a theramutein in a cell as a known substance is a modulator of a corresponding prototheramutein. One embodiment of the method involves contacting a control cell that expresses the prototheramutein and is capable of exhibiting a responsive phenotypic characteristic (linked to the functioning of the prototheramutein in the cell) with the known modulator of the prototheramutein, contacting a test cell that expresses the theramutein and is also capable of exhibiting the responsive phenotypic characteristic

(linked to the functioning of the theramutein in the cell) with the chemical agent, and comparing the response of the treated test cell with the response of the treated control cell; to determine that the chemical agent is at least as effective a modulator of the theramutein as the known substance is a modulator of the prototheramutein. In certain other embodiments, one type of control cell may not express the prototheramutein at all. In other embodiments, the control cell may express about the same amount of the prototheramutein as the test cell expresses of the theramutein. In still other embodiments, the control cell may be capable of exhibiting the responsive phenotypic characteristic to about the same extent as the test cell under certain conditions.

[0014] Theramuteins of the invention that are of particular interest are those involved in regulatory function, such as enzymes, protein kinases, tyrosine kinases, receptor tyrosine kinases, serine threonine protein kinases, dual specificity protein kinases, proteases, matrix metalloproteinases, phosphatases, cell cycle control proteins, docking proteins such as the IRS family members, cell-surface receptors, G-proteins, ion channels, DNA- and RNA-binding proteins, polymerases, and the like. No limitation is intended on the type of theramutein that may be used in the invention. At the present time, three theramuteins are known: BCR-ABL, c-Kit, and EGFR.

[0015] Any responsive phenotypic characteristic that can be linked to the presence of the theramutein (or prototheramutein) in the cell can be employed for use in the method, including, for example, growth or culture properties, the phosphorylation state (or other modification) of a substrate of the theramutein, and any type of transient characteristic of the cell, as will be defined and discussed in detail

#### DESCRIPTION OF THE FIGURES

[0016] Figure 1 shows the effect on growth and viability of different concentrations of Compound 2 (C2) for non-transformed vector control Ba/F3 cells (which are IL-3 dependent) as well as Ba/F3 control cells expressing the "wild type" p210<sup>Bcr-Abl</sup> (designated p210<sup>Bcr-Abl-wt</sup>), and Ba/F3 cells expressing the p210<sup>Bcr-Abl-T315I</sup> drug resistant mutant. Cell counts and viability were determined on a automated cell counter as discussed in detail in the specification. Cell counts are shown by the solid color bars; cell viability is shown by the hashed bars. Note that STI-571 potently inhibits growth of the P210 cell line (yellow bar) whereas it is unable to inhibit the growth of the T315I cell line (red bar) even at 10  $\mu$ M concentration. 500 nM C2 shows the largest specificity gap within this dose-response series.



Compare STI-571 at 10  $\mu$ M to C2 at 500 nM on the T315I cell line (red bars).

Abbreviations: DMSO: dimethylsulfoxide (solvent used for drug dissolution).

[0017] Figure 2 shows the effect on growth and viability of different concentrations of Compound 6 (C6) for non-transformed vector control Ba/F3 cells as well as Ba/F3 cells expressing the p210<sup>Bcr-Abl-T315I</sup> drug resistant mutant. Note that STI-571 potently inhibits growth of the P210 cell line (yellow bar) whereas it is unable to inhibit the growth of the T315I cell line (red bar) even at 10  $\mu$ M concentration. 370 nM C6 shows the largest specificity gap within this dose-response series and was the most potent compound identified in the screen. Compare STI-571 at 10  $\mu$ M to C6 at 370 nM on the T315I cell line (red bars). All other details are as per Fig. 1

[0018] Figure 3 shows various determinations of the specificity gap by comparing the effects of various compounds identified in the screen in terms of their effects on the prototheramutein- and theramutein-expressing cell lines. Compound 3 (C3) shows the best example of the ability of the method to identify a compound that exerts an even greater effect on the theramutein than on its corresponding prototheramutein. (Panel E). Panel A: control DMSO treatments; B: negative heterologous specificity gap; C: slightly positive heterologous specificity gap; D: large positive homologous specificity gap; E: positive heterologous specificity gap. See text for explanations.

[0019] Figure 4 shows autoradiograph of recombinant P210 Bcr-Abl wild type and T315I mutant kinase domains assayed for autophosphorylation activity. 200 ng of protein were preincubated with test substances for 10 minutes under standard autophosphorylation reaction conditions and then radiolabelled ATP was added and the reactions proceeded for 30 minutes at 30°C, after which the samples were separated by SDS-PAGE. The gels were silver-stained, dried down under vacuum and exposed to X-ray film. Note that whereas 10  $\mu$ M STI 571 is effective against wild type P210 Bcr-Abl, it is virtually ineffective against the T315I kinase domain, even at concentrations up to 100  $\mu$ M. C2 and C6 are the best two compounds identified, followed by C5, C7 and C4. All of the compounds tested positively to some extent.

#### DETAILED DESCRIPTION OF THE INVENTION

[0020] A "mutein" is a protein having an amino acid sequence that is altered as a result of a mutation that has occurred in its corresponding gene (Weigel et al, 1989). Such mutations may result in changes in one or more of the characteristics of the encoded protein.

For example, an enzyme variant that has modified catalytic activity resulting from a change in one or more amino acids is a mutein.

[0021] This invention is concerned with proteins harboring an alteration of at least one amino acid residue (the terms "amino acid sequence change" or "amino acid sequence alteration" include changes, deletions, or additions, of at least one amino acid residue, or any combination of deletions, additions, changes) such that the resulting mutein has become (as a result of the mutation) resistant to a known therapeutic agent relative to the sensitivity of the non-mutated version of said protein to the therapeutic agent. This specialized class of muteins is hereinafter referred to as a *theramutein*, and the corresponding protein lacking the mutation is referred to herein as a *prototheramutein*.

[0022] As used herein, "prototheramutein" refers to an endogenously occurring protein in a cell or organism that is susceptible to mutation that confers relative insensitivity (i.e. resistance) to a therapeutic compound which otherwise inhibits or activates the protein. Accordingly, "theramutein" refers to an endogenously occurring protein or portion of a protein in a cell or organism that contains at least one amino acid sequence alteration relative to an endogenous form of the protein, wherein the amino acid sequence change is or was identified or becomes identifiable, or is shown to be clinically significant for the development and progression of a given disease, *following exposure of at least one human being to a substance that is known to inhibit or activate the prototheramutein*. Thus, *by definition*, a theramutein is limited to a protein which harbors a mutation in its corresponding endogenous gene that has been shown to be associated with clinical resistance to a drug that is normally able to activate or inhibit the non-mutated protein (i.e. the prototheramutein; see below). Accordingly, it is apparent to a skilled artisan that, as the genes which encode theramuteins are limited to endogenously occurring, the definition of a theramutein excludes proteins encoded by disease-causing infectious agents such as viruses and bacteria, which would be inappropriate for use in this method. As used herein, the term "endogenous gene" refers to a gene that has been present in the chromosomes of the organism since inception.

[0023] In one aspect of the invention, a theramutein is a protein that is altered for the first time with respect to a commonly occurring "wild type" form of the protein (i.e. the prototheramutein). In another aspect of the invention, a theramutein is a variant of a protein (prototheramutein) that is, itself, already a mutein. In still another embodiment, a theramutein may be further mutated as compared to a previously existing theramutein. In

such instances, the first theramutein (such as the T315I mutant of p210 BCR-ABL (see below), may be thought of as a "primary" theramutein, whereas subsequent mutations of the (already mutated) T315I variant may be termed a secondary theramutein, tertiary theramutein, etc. As exemplified below, a mutein of the invention is a variant of Bcr-Abl tyrosine kinase that escapes inhibition by an inhibitor of the "wild type" Bcr-Abl. Such a Bcr-Abl mutein is altered with respect to a more common or "wild type" form of Bcr-Abl (which is also a mutein as well) in such a way that a property of the protein is altered.

[0024] It will be understood that a mutein of primary interest is a theramutein that may have the same, increased, or decreased specific activity relative to its prototheramutein, and that it is not inhibited or is poorly inhibited by an agent that is capable of inhibiting the prototheramutein. Likewise, another theramutein of primary interest is one that has the same, increased or decreased specific activity (relative to its prototheramutein) and that is not activated or is poorly activated by an agent that is capable of activating the prototheramutein. Other variations are obvious to the skilled artisan. It will be further appreciated that theramuteins can include naturally occurring or commonly observed variants of a protein, for example, variants that are expressed from different alleles of a particular gene. Such variants may be unremarkable with respect to their normal cellular function, with functional differences becoming apparent only in the presence of agents that differentially inhibit or activate the cellular function of the variants. For example, naturally occurring variant of a particular enzyme may have activity profiles that are not substantially different, but a therapeutic agent that modulates one may be ineffective in modulating the other.

[0025] It will be appreciated that, whereas one aspect of the invention is the identification of agents that are active against a theramutein that arises or becomes dominant (by any mechanism) during the course of a treatment, another aspect is the identification of agents that are active against muteins that are common within a population of undiseased individuals, but that are less susceptible to modulation by an approved drug, and where the variation in the activity profile of the mutein becomes important (and is therefore first identified as being a theramutein) only in a disease state such as where it is overexpressed or participates in a signaling process which has otherwise become misregulated. For example, a neoplastic disease may be caused by abnormal regulation of a cellular component other than the theramutein or its prototheramutein, and still be treatable with an inhibitor of the prototheramutein, whereas the same treatment would be less effective or ineffective where

the theramutein was present. This can be an issue where it is observed that the response of a particular tumor type to an anticancer agent varies among individuals that express different variants of an enzyme against which the anticancer agent is directed (Lynch et al., 2004). Here, the variants would not have arisen or become predominant during the course of treatment of the disease, but are preexisting in the healthy population and are detected only by their altered responsiveness to a particular course of established therapeutic treatment.

[0026] As used herein, the terms “agonist” and “activator” of a protein are used interchangeably. An activator (agonist) is limited to a substance that binds to and activates the functioning of a given protein. Unless explicitly stated otherwise, an “activator”, an “agonist”, and an “activator of a protein” are identical in meaning. The activation by an activator may be partial or complete. Likewise, as used herein, the terms an “antagonist” and “inhibitor” of a protein are used interchangeably. An inhibitor (antagonist) is limited to a substance that binds to and inhibits the functioning of a given protein. Unless explicitly stated otherwise, an “inhibitor”, an “antagonist” and an “inhibitor of a protein” are also synonymous. The inhibition by an inhibitor may be partial or complete. A modulator is an activator or an inhibitor. By way of example, an “activator of PKC” should be construed to mean a substance that binds to and activates PKC. Similarly, an “inhibitor of p210<sup>Bcr-Abl</sup>” is a substance that binds to and inhibits the functioning of p210<sup>Bcr-Abl</sup>. To state that a substance “inhibits a protein” means that the substance binds to the protein in order to exert it inhibitory effect. Similarly, to state that a substance “activates protein X” is to state that the substance binds to and activates protein X. The terms “binds,” “binding,” and “binds to” have their ordinary meanings in the field of biochemistry in terms of describing the interaction between two substances (*e.g.*, enzyme-substrate, protein-DNA, receptor-ligand, etc.). As used herein, the term “binds to” is synonymous with “interacts with” in the context of discussing the relationship between a substance and its corresponding target protein.

[0027] The concept of inhibition or activation of a mutated form of an endogenous protein to an equivalent or greater extent than the corresponding non-mutated counterpart protein is defined for the first time and referred to herein as a positive “*specificity gap*.” In general terms, *and using an inhibitor case as an example*, the *specificity gap* refers to the difference between the ability of a given substance, under comparable conditions to inhibit the theramutein in a cell-based assay system as compared to either:

- a) the ability of the same substance under comparable conditions to inhibit the prototheramutein, or
- b) the ability of a second substance (usually a known inhibitor or of the prototheramutein), to inhibit the theramutein under comparable conditions, or
- c) the ability of the second substance to inhibit the prototheramutein under comparable conditions.

[0028] When the comparison is made between the effects of two distinct substances (tested individually) on the theramutein alone, the result is termed an *homologous specificity gap* determination.

[0029] Alternatively, when a comparison is made between the effects of two distinct substances, one of which is tested on the theramutein and the other on the prototheramutein, respectively, the result is termed a *heterologous specificity gap* determination. Thus, (a) and (c) as given above are examples of heterologous SG determinations, whereas (b) is an example of an homologous SG determination.

[0030] Reference to Figure 3 is informative in understanding and elucidating these concepts.

[0031] Analogous issues apply when the case concerns an activator. It will be immediately obvious to the skilled artisan that the term comparable conditions refers to testing two different compounds, for example, at the same concentration, or by comparing IC50 values determined for two distinct compounds on the corresponding theramutein and prototheramutein, etc.

[0032] Thus, in one embodiment of the application of this approach, substances that are more effective against a theramutein have a "positive specificity gap." A "zero, null or no" specificity gap indicates that there is no significant measurable difference between the effect of a substance on the theramutein as compared to the prototheramutein, (however such compounds may be quite useful in their ability to inhibit or activate both a theramutein and its corresponding prototheramutein) and a "negative specificity gap" indicates a drug or other substance that at a given concentration is less effective against the theramutein than against the unmutated endogenous form. The latter category is generally of lesser interest than the former categories of compounds, except in the case where the compound is so potent that its lesser effect on the theramutein is of no real concern. The skilled investigator can easily

recognize a variety of approaches to quantifying the specificity gap assessment in a manner tailored to his or her needs.

[0033] The invention provides a means for identifying compounds that exhibit a desired specificity gap. Such compounds can be identified and their ability to inhibit or activate the theramutein determined using an *in vitro* cell-based assay system where the effect of a drug on the cellular functioning of the mutated endogenous form of the protein is compared to the effect of the same drug on the cellular functioning of a non-mutated endogenous form of the protein.

[0034] Thus, the system enables the discovery of compounds capable of binding to a theramutein and exerting a greater modulatory effect on the cellular functioning of a theramutein than on its corresponding prototheramutein. Further, the system enables the discovery of compounds capable of binding to a theramutein and exerting at least as great or greater modulatory effect on the cellular functioning of a theramutein than previously known compounds are able to exert on the corresponding prototheramutein. In a particular embodiment of the invention, a compound may be screened for and identified that 1) is at least as effective against the theramutein as the original drug is against the prototheramutein, and 2) is similarly effective against the prototheramutein as against the theramutein (*i.e.*, displays a small or essentially zero specificity gap).

[0035] In an embodiment of the invention, cells that overexpress a theramutein of interest are used to identify chemical agents that are inhibitors or activators of (*i.e.*, that bind to and inhibit or that bind to and activate) at least the selected theramutein. The chemical agents may also be inhibitors or activators of the prototheramutein or even other theramuteins of the same prototheramutein. As used herein, the terms "chemical agent" and "compound" are used interchangeably, and both terms refer exclusively to substances that have a molecular weight up to about 2000 atomic mass units (Daltons). Such substances are sometimes referred to as "small molecules." Unless otherwise stated herein, the term substance as used herein also refers only to chemical agents/compounds, and does not refer to *biological agents*. As used herein, "*biological agents*," are molecules which include proteins, polypeptides, and nucleic acids, and have molecular weights at or above 2001 atomic mass units (Daltons).

[0036] According to the invention, a theramutein is selected and used in a cell-based assay system designed to identify agents that are inhibitors of the theramutein. Where two or

more distinct theramuteins are known, it is strongly advisable to select the most resistant theramutein available for use in the assay system. In general, the degree of resistance of a theramutein is determined relative to its non-mutated counterpart (prototheramutein) using the drug or other substance that was first administered and known to inhibit or activate the prototheramutein and against which the theramutein "arose." The methods of determining the degree of such resistance, for example by analysis of  $IC_{50}$  or  $AC_{50}$  values, are well known in the art and will not be reiterated herein. However, no causal relationship is necessary or should be inferred between the treatment of the patient with a given therapeutic agent and the appearance of a theramutein. Rather, what is required in order to practice the invention is that a true theramutein be properly selected according to the teachings herein.

[0037] Thus, for example, randomly generated site directed mutants of known proteins that are created in the laboratory but that have *not* been shown to be clinically relevant are not appropriate muteins for use within the scope of this invention. Such muteins would not, of course, be properly classified as theramuteins

[0038] For example, in an effort to obtain potential inhibitors of mutants of p210<sup>Bcr-Abl</sup>, Huron et al. used a recombinant c-abl preparation and screened a series of compounds known to inhibit c-src tyrosine kinase activity. The authors performed c-abl kinase assays on their compounds and identified the most potent compound as an 8 nM inhibitor against c-abl. When this compound (PD166326) was tested against various p210<sup>Bcr-Abl</sup> theramuteins, however, it showed activity against some of the mutants such as p210<sup>Bcr-Abl-E255K</sup>, but the p210<sup>Bcr-Abl-T315I</sup> theramutein was found to remain 10 fold more resistant (Huron et al. 2003, Table 3). Furthermore, in each case the compound was still markedly *less effective* on the p210<sup>Bcr-Abl</sup> theramuteins than it was against the wild-type p210<sup>Bcr-Abl</sup>. When the compound was tested against p210<sup>Bcr-Abl-T315I</sup> mutant activity, it was unable to inhibit the activity to any appreciable extent (p. 1270, left hand column, second paragraph; see also Fig. 4.). Thus, the disclosed compound was able to inhibit a theramutein that is partially resistant to STI-571, but had no activity against the T315I mutant of Bcr-Abl., which is known to be the theramutein that exhibits the most resistance to STI-571 (imatinib mesylate; Gleevec).

[0039] Indeed, prior to this invention, no one has been successful in identifying a chemical agent, or even a methodology, capable of identifying a chemical agent that effectively inhibits the p210<sup>Bcr-Abl-T315I</sup> theramutein to an equal or greater extent than STI-571 (imatinib mesylate; Gleevec) is able to do with respect to the wild type p210<sup>Bcr-Abl</sup> protein.

Such compounds would be immensely useful, because at the present time there is no alternative for patients who progress to STI-571 resistant status. Once patients develop resistance to STI-571, there is no other effective alternative treatment available, and unfortunately death is certain.

[0040] In the present invention, a test cell is used that displays a phenotypic characteristic (as defined below) which is linked to the presence and functional activity of the theramutein in the cell under appropriate conditions. This can be the same as a phenotypic characteristic displayed by a cell that expresses the prototheramutein. A phenotypic characteristic (i.e. a non-genotypic characteristic of the cell) is a property which is observed (measured), selected and/or defined for subsequent use in an assay method as described herein. Expression of the phenotypic characteristic depends on (or is responsive to) the total activity of the theramutein in the cell, and is a product of the absolute amount of the theramutein and its specific activity. Often, the phenotypic characteristic is observable as a result of elevated levels of theramutein activity and is not apparent in cells that express low amounts of the theramutein or its prototheramutein. Further, it can be demonstrated that the phenotypic characteristic is modulated by modulating the specific activity of the theramutein with an inhibitor or activator of the theramutein. In this manner, the selected phenotypic characteristic is linked to the presence of the theramutein in the test cell. Notably, for theramuteins, the selected phenotypic characteristic is usually also displayed by a cell that overexpresses the prototheramutein and in which the phenotypic characteristic is modulated by known inhibitors or activators of the prototheramutein.

[0041] A phenotypic characteristic is simply a characteristic of a cell or organism other than a genotypic characteristic of the cell or organism. No other limitation of the term phenotypic characteristic of any kind or nature is intended or appropriate in order to properly and effectively practice the invention. The skilled artisan must be able to select any characteristic of the cell that maximizes the utility of establishing the proper cell-based assay for his or her needs. The phenotype characteristic can be quantitative or qualitative and be observable or measurable directly (*e.g.*, observable with the naked eye or with a microscope), but most commonly the characteristic is measured indirectly by assay using standard automated laboratory equipment and procedures. The term "observable" means that a characteristic may be measured or is otherwise detectable under appropriate conditions by any means whatsoever, including the use of any type of laboratory instrumentation available.



The term "detectable" is not the same as "detected". A characteristic may be detectable to a skilled artisan without being detected at any given time, depending upon how the investigator chooses to design the assay system. For example, in searching for activators of a prototheramutein (or theramutein), it may be desirable to have the relevant phenotypic characteristic detected only after the addition of a known activator or test substance capable of activating the POI. This provides the ability to maximize the intensity of the signal that is generated by the test cell in the assay.

[0042] Phenotypic characteristics include but are not limited to growth characteristics, transformation state, differentiation state, substrate phosphorylation state, catalytic activity, ion flux across the cell membrane (calcium, sodium, chloride, etc.), pH changes, fluctuations of second messenger molecules or other intracellular chemical species such as cAMP, phosphoinositides, cyclic nucleotides, modulations of gene expression, and the like. The characteristic of the cell may be observable or measureable continuously (*e.g.*, growth rate of a cell), or after a period of time (*e.g.*, terminal density of a cell culture), or transiently (*e.g.*, modulation of a mutein causes a transient change in phosphorylation of a substrate of the mutein, or a transient flux in ion flow across the membrane, or elevations or reductions in intracellular cAMP levels). In certain embodiments, a selected phenotypic characteristic may be detectable only in the presence of a modulator of the prototheramutein or the theramutein. For example, a phenotypic characteristic can be focus formation that becomes observable when a cell that overexpresses a selected protein is cultured in the presence of an activator of the protein, or it may be a transient increase or decrease in the level of an intracellular metabolite or ion, such as cAMP, Calcium, Sodium, phosphoinositol, cGMP, etc.

[0043] The term "responsive phenotypic characteristic" means a characteristic of the cell which is responsive to inhibitors or activators of a given protein (prototheramutein or theramutein). The term "known therapeutic agent" is defined as any agent that has been administered to a human being for the treatment of a disease in a country of the world.

[0044] A useful phenotypic characteristic, as exemplified herein in association with p210<sup>Bcr-Abl</sup> and theramuteins thereof, is dysregulation of cell growth and proliferation. It is noted that the same or similar assay may be appropriate for use with many different proteins of interest. For example, dysregulations of growth, proliferation, and/or differentiation are common phenotypic characteristics that may result from overexpression of a variety of different cellular proteins. By overexpressing a selected protein in order to cause the

appearance of such a phenotypic characteristic, the phenotypic characteristic becomes linked to the presence, amount, and specific activity of that selected protein under suitable conditions.

[0045] Though not always necessary, it will often be advantageous to employ cells that express high levels of the theramutein, and to select a phenotypic characteristic that results from overexpression of the theramutein. This is because phenotypic characteristics become more distinguishable (easier to measure) as a theramutein is overexpressed to a greater extent. Further, changes (or responses) of the phenotypic characteristic that are observed in response to modulators of the theramutein are often amplified as the functional level of the theramutein is increased. Expressed another way, the selected (responsive) phenotypic characteristic observed in cells that overexpress the theramutein is particularly sensitive to modulators of the theramutein. The theramutein should be stably expressed. Stable expression means that the level of the protein in the cell does not change substantially during the course of an assay. For example, stimulation or activation of a component of a signaling pathway may be followed by a refractory period during which signaling is inhibited due to down-regulation of the component. For theramuteins of the invention, such down-regulation is usually sufficiently overcome by artificially overexpressing the theramutein. Expressed another way, the expression is sufficiently maintained that changes in a phenotypic characteristic that are observed during the course of an assay are due primarily to inhibition or activation of the theramutein. For these reasons, although stable expression of the theramutein is preferred, transfection followed by transient expression of the theramutein may be employed provided that the duration of the assay system is short relative to the progressive decline in the levels of the transiently expressed theramutein that inevitably result from such systems.

[0046] A preferred drug screening method of the present invention involves the following:

[0047] 1) Identification of a theramutein for which a novel inhibitor or activator is desired. Identification of an appropriate theramutein may be performed using standard techniques (Gorre et al., Science; see also PCT/US/02/18729). Briefly, patients that have been given a course of therapeutic treatment using an activator or inhibitor of a known or suspected prototheramutein and have shown clinical signs and symptoms consistent with disease relapse are identified, and cells or tissue samples derived from such patients are

obtained. Using standard laboratory techniques such as RT-PCR, the sequence of the prototheramutein is determined and compared to the previously determined nucleic acid sequence of the known prototheramutein gene or cDNA sequence. Mutations, if present, are identified and are correlated with functional resistance of the prototheramutein's function either in cell-based or, more commonly, cell-free assay systems, again using standard methodology. Once resistance-inducing mutations are confirmed, then said one or more confirmed mutants comprise a defined theramutein which may be used in the subsequent methods as described herein.

[0048] 2) Provision of a test cell that expresses a theramutein of interest and displays an observable (measurable) phenotypic characteristic which is responsive to inhibitors or activators of the theramutein. In one embodiment, a cell is selected that naturally expresses the theramutein such that a responsive phenotypic characteristic is present under suitable culture conditions which are obvious to one of ordinary skill in the art. In other embodiments, the theramutein is overexpressed, in some instances in a host cell that does not otherwise express the theramutein at all. This usually involves construction of an expression vector from which the theramutein can be introduced into a suitable host cell and overexpressed using standard vector systems and methodology. In one embodiment, overexpression results in a level of the theramutein that is at least about 3 times the amount of the protein usually present in a cell. Alternatively, the amount is at least about 10 times the amount usually present in a cell. In another embodiment, the amount is at least about 20 times or more preferably at least about 50 times the amount usually present in a cell.

[0049] 3) Provision of a control cell that expresses the prototheramutein of the theramutein of interest. As muteins described herein usually retain catalytic activity, the control cell displays substantially the same phenotypic characteristic as the test cell. The phenotypic characteristic need not be quantitatively alike in both cells. For example, a mutation that leads to reactivation of the prototheramutein may also increase, decrease, or otherwise affect its specific activity with respect to one or more of its substrates in the cell. As a result, it may exhibit the selected phenotypic characteristic to a greater or lesser extent. Accordingly, it may be desirable in some cases to adjust expression of either or both of the prototheramutein and the theramutein such that test and control cells exhibit the phenotypic characteristic to approximately the same degree. This may be done, for example, by

expressing the proteins from promoters whose activity can be adjusted by adjusting the amount of inducer present, all using standard methodology (see, for example, Maniatis et al.)

[0050] The skilled investigator may also wish to use unmodified host cells or host cells harboring the expression vector only as control cells for certain experimental procedures. (The host cells are the cells into which an expression vector encoding the theramutein was introduced in order to generate the test cells.

[0051] 4) The test and control cells are then cultured (although not necessarily at the same time) in growth media (or even in intact animals) under suitable conditions such that the selected phenotypic characteristic can be expressed and assayed. Control cells that are expressing the prototheramutein are incubated with a known modulator of the prototheramutein, or with a test substance, and test cells are incubated with test compounds to determine whether they are active against the theramutein, as measured by the ability of said substances to affect the selected phenotypic characteristic in the expected manner. Alternatively, control cells not expressing the prototheramutein may also be substituted, depending upon the particular characteristic of the cell that the skilled investigator has chosen for study. Substances may then be assayed on the test cells and, optionally, on the control cells at the same time, or at another time, and the results compared.

[0052] In one embodiment of the invention, substances that are active with regard to the test cells can be rapidly identified by their ability to modulate the phenotypic response of the test cells in the same manner as, for example, the known modulator of the prototheramutein alters the phenotypic characteristic of the control cells. In another embodiment, active substances may be identified by their ability to modulate the activity of the theramutein in the test cells while having little or no effect on the unmodified (prototheramutein non-expressing) control cells. The skilled investigator will readily appreciate the many variations of this approach that may be utilized to identify, for example, modulators that are more effective against the theramutein, or that are equally effective against both the prototheramutein and the theramutein.

[0053] Other responsive phenotypic characteristics can be observed and/or measured and include, for example, detection of substrates of the prototheramutein, and detection of gene expression changes that are regulated by the activity of the theramutein. In the simplest terms, any characteristic of the cell that the skilled investigator has previously correlated with the functional activity of the theramutein may be suitable for use with such methods. These

methods include detection of fluorescence of suitably labeled proteins (FACS), immunohistochemistry (IHC) for detection of protein expression, competitive radioligand binding assays, solid matrix blotting techniques, such as Northern and Southern blots of cell extracts, reverse transcriptase polymerase chain reaction (RT-PCR) and ELISA. The phenotypic characteristic may be detected either on the intact cell after treatment with a test substance or, alternatively, on a subcellular fraction of the test or control cells after treatment with a test substance.

[0054] Once compounds are identified that have the desired effect on the theramutein expressing test cells, it is desirable (but not necessary) to independently verify that the compounds identified are exerting their effects on the theramutein through a direct binding mechanism, i.e. that the compounds fulfill the criteria of being inhibitors or activators (as desired) of the theramutein. This may be accomplished with numerous standard binding assays that are known to one of ordinary skill in the art and will not be reiterated here. Numerous reference texts comprehensively discuss such techniques (see, for example, Foreman and Johansen, 2002; Enna S.J. et al. (1991) *Current Protocols in Pharmacology*, Wiley & Sons, Incorporated; Bonifacino, J.S. et al. (1999) *Current Protocols in Cell Biology*, Wiley & Sons, Incorporated).

[0055] In a particular embodiment of the invention, the method is used to identify substances that are inhibitors of the p210<sup>Bcr-Abl-T315I</sup> theramutein. The prototheramutein and theramutein are each expressed in Ba/F3 (murine) cells using standard methodology and the responsive phenotypic characteristics that are observed are growth characteristics (terminal cell density for a carefully defined cell culture, and growth in the absence of Interleukin-3 (IL-3)).

[0056] Another useful assay is the determination of the state of phosphorylation of a direct substrate of p210<sup>Bcr-Abl-T315I</sup>. One such substrate is Crkl (Gorre et al., *Science* 293:876-80 (2001)), an adapter protein which mediates the connection between Bcr-Abl and Ras. The phosphorylation state of CRKL is representative of the signaling activity of p210<sup>Bcr-Abl</sup> in a cell. Another downstream substrate is p62DOK. Any such substrate would suffice for these purposes.

[0057] As exemplified herein, inhibitors of the T315I theramutein have been identified. Furthermore, these inhibitors are also active to differing extents against the wild type prototheramutein p210<sup>Bcr-Abl-wt</sup>.

[0058] According to the present invention, a therapeutically effective amount of one or more compounds that modulate the functional activity of a p210<sup>Bcr-Abl</sup> theramutein is administered to a mammal in need thereof. The term "administering" as used herein means delivering the compounds of the present invention to a mammal by any method that may achieve the result sought. They may be administered, for example, orally, parenterally (intravenously or intramuscularly), topically, transdermally or by inhalation. The term "mammal" as used herein is intended to include, but is not limited to, humans, laboratory animals, domestic pets and farm animals. "Therapeutically effective amount" means an amount of a compound that, when administered to a mammal, is effective in producing the desired therapeutic effect, such as inhibiting kinase activity, inhibiting cancer cell growth and division, etc.

[0059] The invention provides a method of treating disease in a mammal by administering to the mammal an effective amount of a modulator of a theramutein. Suitable diseases to be treated according to the present invention include, but are not limited to, relapsing neoplastic or other proliferative disorders that have become resistant to previously administered drugs. The method is also useful for overcoming variation among individuals with respect to susceptibility to drug treatment that results from allelic differences among therapy targets. For example, the role of p210<sup>Bcr-Abl</sup> tyrosine kinase signaling in CML has been extensively demonstrated, as has the role of theramuteins of p210<sup>Bcr-Abl</sup> in drug resistant recurrence of CML. Further, different muteins of p210<sup>Bcr-Abl</sup> exhibit varying sensitivity to inhibitors of p210<sup>Bcr-Abl</sup>. Although some theramuteins arise during drug therapy, others may be preexisting in the population. These latter examples will not be recognized as theramuteins until such time as the disease state ensues and is followed by treatment with a known class of therapeutic agents. Only after said treatment will such preexisting theramuteins reveal themselves as being clinically significant in terms of relative non-responsiveness leading to the progression of the disease in the patient harboring the theramutein.

[0060] In an embodiment of the invention, theramutein modulators are administered in combination with one or more other anti-neoplastic agents. Any suitable anti-neoplastic agent can be used, such as a chemotherapeutic agent, radiation or combinations thereof. The anti-neoplastic agent can be an alkylating agent or an anti-metabolite. Examples of alkylating agents include, but are not limited to, cisplatin, cyclophosphamide, melphalan, and

dacarbazine. Examples of anti-metabolites include, but not limited to, doxorubicin, daunorubicin, and paclitaxel, gemcitabine, and topoisomerase inhibitors irinotecan (CPT-11), aminocamptothecin, camptothecin, DX-8951f, and topotecan (topoisomerase I) and etoposide (VP-16) and teniposide (VM-26) (topoisomerase II). When the anti-neoplastic agent is radiation, the source of the radiation can be either external (external beam radiation therapy – EBRT) or internal (brachytherapy – BT) to the patient being treated. The dose of anti-neoplastic agent administered depends on numerous factors, including, for example, the type of agent, the type and severity of the tumor being treated and the route of administration of the agent. It should be emphasized, however, that the present invention is not limited to any particular dose, route of administration, or combination of chemotherapeutic agents or other therapeutic regimens that are combined with the administration of theramutein modulators.

[0061] Anti-neoplastic agents which are presently known in the art or being evaluated can be grouped into a variety of classes including, for example, mitotic inhibitors, alkylating agents, anti-metabolites, intercalating antibiotics, growth factor inhibitors, cell cycle inhibitors, enzymes, topoisomerase inhibitors, anti survival agents, biological response modifiers, anti-hormones, and anti-angiogenesis agents, all of which can be administered with inhibitors or activators of theramuteins.

[0062] Modulators of theramuteins can be administered with antibodies that neutralize other receptors involved in tumor growth. In an embodiment of the invention, a theramutein modulator is used in combination with a receptor antagonist that binds specifically to the Epidermal Growth Factor Receptor (EGFR). Particularly preferred are antigen-binding proteins that bind to the extracellular domain of EGFR and block binding of one or more of its ligands and/or neutralize ligand-induced activation of EGFR. An EGFR antagonist can be an antibody that binds to EGFR or a ligand of EGFR and inhibits binding of EGFR to its ligand. Ligands for EGFR include, for example, EGF, TGF- $\alpha$ , amphiregulin, heparin-binding EGF (HB-EGF) and betacellulin. EGF and TGF- $\alpha$  are thought to be the main endogenous ligands that result in EGFR-mediated stimulation, although TGF- $\alpha$  has been shown to be more potent in promoting angiogenesis. It should be appreciated that the EGFR antagonist can bind externally to the extracellular portion of EGFR, which can or can not inhibit binding of the ligand, or internally to the tyrosine kinase domain. Examples of EGFR antagonists that bind EGFR include, without limitation, biological agents such as antibodies (and functional equivalents thereof) specific for EGFR, and chemical agents (small

molecules), such as synthetic kinase inhibitors that act directly on the cytoplasmic domain of EGFR.

[0063] Other examples of growth factor receptors involved in tumorigenesis are the receptors for vascular endothelial growth factor (VDGFR-1 and VEGFR-2), platelet-derived growth factor (PDGFR), nerve growth factor (NGFR), fibroblast growth factor (FGFR), and others.

[0064] In a combination therapy, the theramutein inhibitor is administered before, during, or after commencing therapy with another agent, as well as any combination thereof, *i.e.*, before and during, before and after, during and after, or before, during and after commencing the anti-neoplastic agent therapy. For example, the theramutein inhibitor can be administered between 1 and 30 days, preferably 3 and 20 days, more preferably between 5 and 12 days before commencing radiation therapy. In a preferred embodiment of the invention, chemotherapy is administered prior to, concurrently with or, more preferably, subsequent to antibody therapy.

[0065] In the present invention, any suitable method or route can be used to administer theramutein inhibitors of the invention, and optionally, to co-administer anti-neoplastic agents and/or antagonists of other receptors. The anti-neoplastic agent regimens utilized according to the invention, include any regimen believed to be optimally suitable for the treatment of the patient's neoplastic condition. Different malignancies can require use of specific anti-tumor antibodies and specific anti-neoplastic agents, which will be determined on a patient to patient basis. Routes of administration include, for example, oral, intravenous, intraperitoneal, subcutaneous, or intramuscular administration. The dose of antagonist administered depends on numerous factors, including, for example, the type of antagonists, the type and severity tumor being treated and the route of administration of the antagonists. It should be emphasized, however, that the present invention is not limited to any particular method or route of administration.

[0066] Suitable carriers include, for example, one or more of water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like, as well as combinations thereof. Carriers can further comprise minor amounts of auxiliary substances, such as wetting or emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the theramutein modulator as the active ingredient. The compositions can, as is well known



in the art, be formulated so as to provide quick, sustained or delayed release of the active ingredient after administration to the mammal.

[0067] The compositions of this invention can be in a variety of forms. These include, for example, solid, semi-solid and liquid dosage forms, such as tablets, pills, powders, liquid solutions, dispersions or suspensions, liposomes, suppositories, injectable and infusible solutions. The preferred form depends on the intended mode of administration and therapeutic application.

[0068] Such compositions of the present invention are prepared in a manner well known in the pharmaceutical art. In making the composition the active ingredient will usually be mixed with a carrier, or diluted by a carrier and/or enclosed within a carrier which can, for example, be in the form of a capsule, sachet, paper or other container. When the carrier serves as a diluent, it can be a solid, semi-solid, or liquid material, which acts as a vehicle, excipient or medium for the active ingredient. Thus, the composition can be in the form of tablets, lozenges, sachets, cachets, elixirs, suspensions, aerosols (as a solid or in a liquid medium), ointments containing, for example, up to 10% by weight of the active compound, soft and hard gelatin capsules, suppositories, injection solutions, suspensions, sterile packaged powders and as a topical patch.

[0069] It should be appreciated that the methods and compositions of the present invention can be administered to any suitable mammal, such as a rabbit, rat, or mouse. More preferably, the mammal is a human.

[0070] Throughout this application, various publications, reference texts, textbooks, technical manuals, patents, and patent applications have been referred to. The teachings and disclosures of these publications, patents, patent applications and other documents in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which the present invention pertains.

[0071] It is to be understood and expected that variations in the principles of invention herein disclosed may be made by one skilled in the art and it is intended that such modifications are to be included within the scope of the present invention.

[0072] The following examples further illustrate the invention, but should not be construed to limit the scope of the invention in any way. Detailed descriptions of conventional methods, such as those employed in the construction of vectors and plasmids, the insertion of genes encoding polypeptides into such vectors and plasmids, the introduction

of plasmids into host cells, and the expression and determination thereof of genes and gene products can be obtained from numerous publications, including Sambrook, J et al., (1989) *Molecular Cloning: A Laboratory Manual*, 2<sup>nd</sup> ed., Cold Spring Harbor Laboratory Press; Coligan, J. et al. (1994) *Current Protocols in Immunology*, Wiley & Sons, Incorporated; Enna, S.J. et al. (1991) *Current Protocols in Pharmacology*, Wiley & Sons, Bonifacino, J.S. et al. (1999) *Current Protocols in Cell Biology*, Wiley & Sons. All references mentioned herein are incorporated in their entirety.

#### EXAMPLES

[0073] p210<sup>Bcr-Abl-T315I</sup> is a theramutein of the p210<sup>Bcr-Abl</sup> protein (p210<sup>Bcr-Abl</sup>) that is resistant to inhibition by imatinib mesylate (Gleevec, STI-571). The mutation at position 315 converts a threonine to an isoleucine residue and is one of several mutations that are observed among resistant or relapsed patients. This particular mutant, however, is the most resistant such theramutein yet identified.

[0074] A responsive phenotypic characteristic was determined for a Ba/F3 cell line engineered to overexpress the p210<sup>Bcr-Abl-T315I</sup> theramutein. The responsive phenotypic characteristic was determined relative to non-transformed Ba/F3 cells and Ba/F3 cells that express the p210<sup>Bcr-Abl-wt</sup> prototheramutein. The responsive phenotypic characteristic was the ability of the T315I mutants to grow to a higher cell saturation density under analogous culture conditions as compared to the control non-transformed Ba/F3 cell line, and to grow in the absence of interleukin 3 (IL-3), which is required for maintenance of the control non-transformed Ba/F3 cell line.

[0075] The detection system utilized was a high speed cell imaging and counting system in which 3 µl sample volumes of cells were sequentially injected through a 5 µl optical microcell, digitally imaged and stored on computer, scanned, and then counted, all under a microcomputer-based control system. The system has the capacity to perform direct cell counts on samples from cultures as small as 500 µl and provides statistically significant total cell counts from culture samples containing as few as 12,500 cells. All of the figures displaying cell count and viability assays utilized this system for data acquisition and analysis. Simultaneously with the cell count performed, the system is also capable of determining overall cell viability by distinguishing counted, imaged cells that have excluded trypan blue (counted as "viable" cells) from cells which have taken up the trypan blue dye (counted as "non-viable" cells). Injection of trypan blue into the cell sample occurs

immediately prior to the sample being sequentially injected into the microcell for simultaneous cell counting and imaging.

[0076] The system may be integrated into the workflow of high-throughput screening devices to provide a sensitive and precise cell counting and cell viability assay system that is more reliable and less prone to confounding effects of metabolic viability-based cellular assays such as XTT or Alamar blue.

[0077] Initially, approximately 113,000 compounds were screened at concentrations ranging from 10 to 20  $\mu$ M to identify a subset that was capable of affecting growth of Ba/F3 cells (Ba/F3 T315I cells) overexpressing the p210<sup>Bcr-Abl-T315I</sup> theramutein by any means.

[0078] A total of approximately 11,760 compounds showed greater than 50% growth inhibition, corresponding to 4557 distinct chemical classes. Retesting of these 4557 compounds with the same cell line yielded a database of compound responsiveness which was then sorted and rank ordered according to those compounds exhibiting the highest overall growth inhibition. From this rank ordered database, the highest scoring 130 compounds were then rescreened in a defined cell-based assay system using Ba/F3 T315I as test cells and wild type Ba/F3 as control cells. Compounds of interest were those that differentially inhibited growth of Ba/F3 cells expressing the p210<sup>Bcr-Abl-T315I</sup> theramutein relative to non-transformed wild type Ba/F3 cells. Six compounds were identified that fulfilled the desired criteria, and some of these compounds were analyzed in further detail using the Ba/F3 p210<sup>Bcr-Abl-wt</sup> cells line (Ba/F3 P210 cells) as well. One compound was unavailable for further testing due to lack of availability of additional material from the chemical supplier. The remaining five compounds were independently evaluated in additional cell-based assays using the aforementioned cell lines as well as in a cell-free purified protein kinase assay using human recombinantly produced 120 Kd kinase domain fragments isolated from both wild type P210 Bcr-Abl as well as P210 T315I mutant kinase domain.

[0079] All five compounds inhibited p210<sup>Bcr-Abl-T315I</sup> 120 Kd activity as measured by inhibition of autophosphorylation activity, as shown in Figure 4. Thus, of the 6 highest scoring compounds out of more than 113,000 compounds screened, at least 5 of the six directly inhibited the p210<sup>Bcr-Abl-T315I</sup> mutant directly. It is noteworthy that Compound 5 appears to spread the recombinant protein band out on the SDS page gel. This was also evident on the silver-stained gel (data not shown). It is possible that this compound may

actually be a "suicide" inhibitor that is able to covalently cross-link the POI in order to permanently inhibit its activity, but this will require further study.

[0080] Taken together, the teachings and the results described herein provide conclusive proof that the system is capable of identifying inhibitors or activators of the selected theramutein, and the skilled investigator will immediately recognize that such a system may be applied to any other theramutein with only obvious, minor modifications.

[0081] Representative examples of the cell-based assay results demonstrating selective inhibition of growth of the Ba/F3 T315I cell line relative to the wild type non-transformed Ba/F3 cells are shown in Figures 1 and 2. The compounds inhibited growth and reduced the viability of cells expressing the T315I theramutein at concentrations under which the growth and viability of the wild type Ba/F3 non-transformed cells (not expressing either p210<sup>Bcr-Abl-wt</sup> or p210<sup>Bcr-Abl-T315I</sup>) were relatively unaffected, whereas cells expressing both the prototheramutein as well as the theramutein were substantially inhibited. In some instances, the T315I expressing cells were inhibited to an even greater extent than the P210 prototheramutein expressing cells. (See, for example, Figure 3, right hand side, Compound 3 results against P210 and T315I cells.

[0082] In summary, the methods presented herein provide a fundamental advance in the form of a generalizable approach for identifying modulators of any given theramutein. The results demonstrate conclusively the power of the method to identify critically needed compounds to overcome a specific type of acquired drug resistance that is uniformly fatal in certain patient populations and is presently untreatable. Furthermore, it is evident to one of skill in this art that the techniques and methods described herein may, using obvious modifications, be straightforwardly generalized to any potential theramutein of clinical significance.

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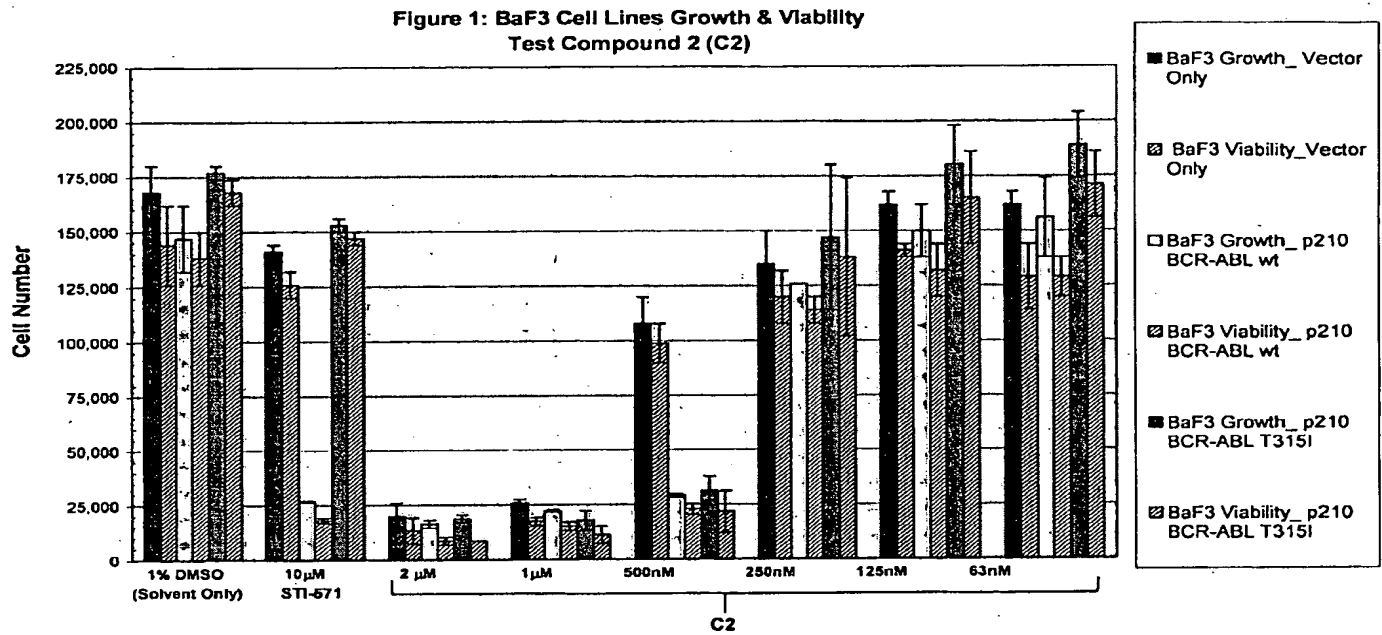


Figure 2: BaF3 Cell Lines Growth & Viability  
Test Compound 6 (C6)

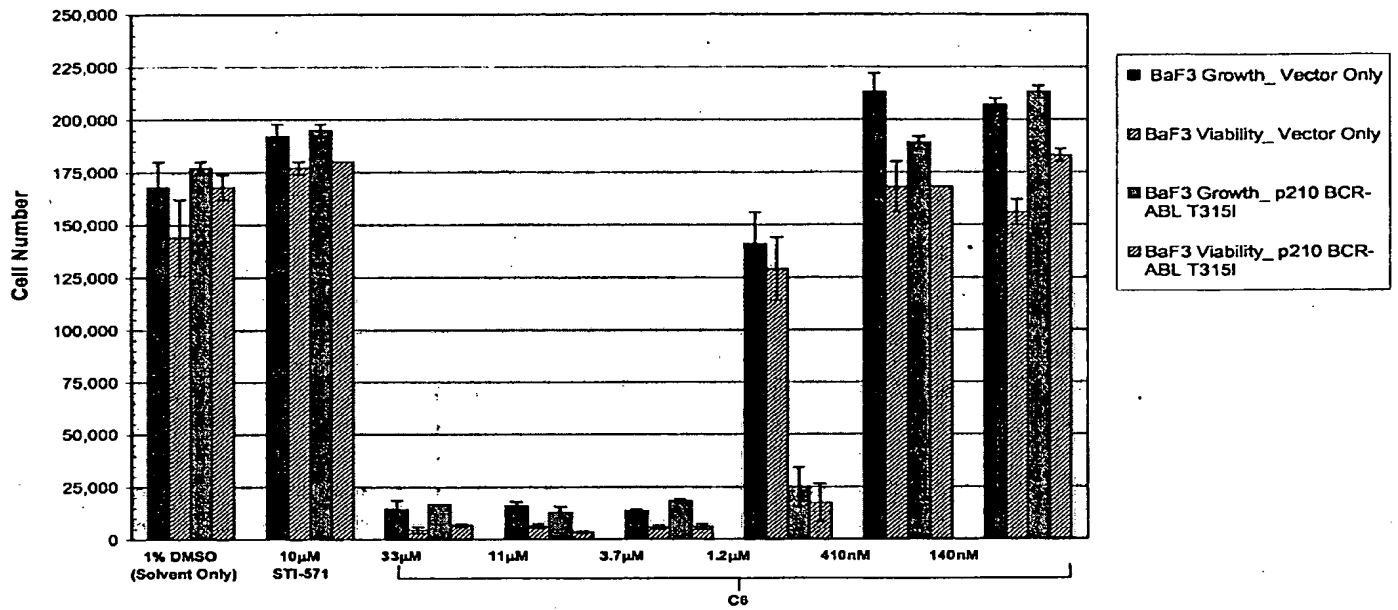


Figure 3: "Specificity Gap" Determinations

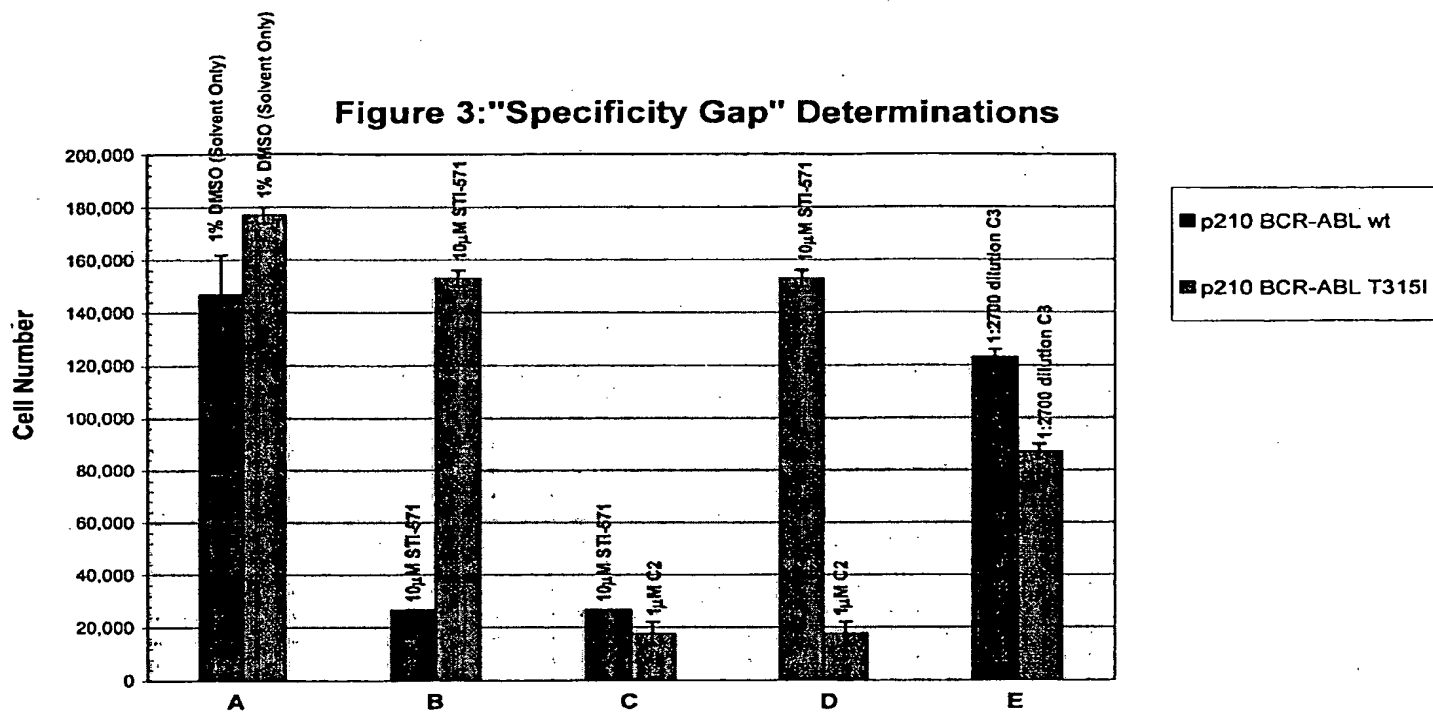
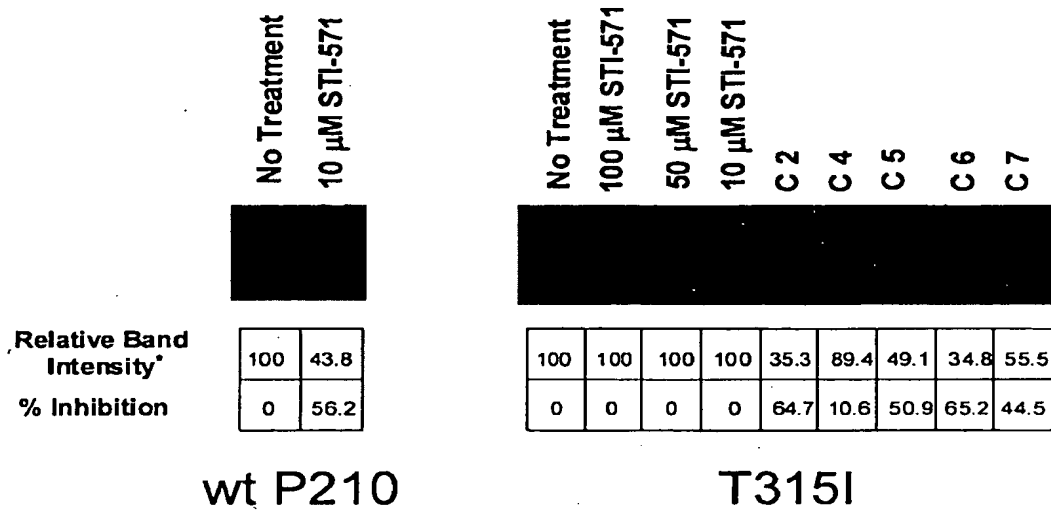


Figure 4: Novel Inhibitors of the Abelson T315I Mutant Kinase



\*Determined by Digital Scanned Image Analysis